Antibodies to anticytotoxic proteins

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T/784

This invention relates to antibodies against the two Tumor Necrosis Factor (TNF) Binding Proteins designated TBP-I and TBP-II which were described in prior patent applications No. 83878 and No. 90339, respectively, of the same applicants. It further relates to the ability of said antibodies to block the binding of TNF to cells, the two antibodies affecting differently cells of different kinds. It relates also to the use of those antibodies and of F(ab) fragments thereof, and salts, functional derivatives or active fractions of said antibodies and fragments, as pharmaceutical agents both for mimicking and blocking effects of TNF on part or all cells in the human body.

Another aspect of the invention features the diagnostic use or tests for measuring either the TBPs or the antisera against them, based on determining the interaction of the antibodies with the TBPs. These diagnostic uses are of two kinds: (a) detecting endogenously produced antibodies to TBPs in body fluids, to determine the extent to which such antibodies, by mimicking or blocking the effects of TNF, contribute to pathological manifestations of diseases, and (b) quantifying the levels of TBPs in body fluids to detect or measure over- or under-production of these proteins in any disorder characterized by abnormal production of said proteins.

The proteins TBP-I and TBP-II, which specifically bind tumor necrosis factor, were isolated from human urine by affinity purification on a column of immobilized TNF, followed by reverse phase high performance liquid chromatography (HPLC). The apparent molecular weights of the two proteins in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were similar (about 30000). Both proteins provided protection against the in vitro cytocidal effect of TNF and both bound lymphotoxin or TNF-β less effectively than tumor necrosis factor or TNF-α. However, the two proteins were found immunologically distinct and had differing N-terminal amino acid sequences: Asp-Ser-Val-Cys-Pro... for TBP-I and Val-Ala-Phe-Thr-Pro... for TBP-II. The N-terminal sequence of TBP-I was homogeneous while that of TBP-II was truncated, to varying extent.

Tumor Necrosis Factor or TNF-α and lymphotoxin or TNF-β (hereinafter, TNF refers to both TNF-α and TNF-β) are cytokines which have many effects on cells. Both TNF-α and TNF-β initiate their effects by binding to specific cell surface receptors. Some of the effects, such as destruction of tumor cells or virus-infected cells, are likely to be beneficial to the organism and to contribute to defense against infectious agents and recovery from injury. However, both TNF-α and TNF-β also have effects which can be extensively deleterious. Over-production of TNF-α can play a major pathogenic role in several diseases. Thus, effects of TNF-α, primarily on the vasculature, are known to be a major cause for symptoms of septic shock. In some diseases, TNF may cause excessive loss of weight (cachexia) by suppressing

activities of adipocytes and by causing anorexia and thus TNF-α was called cachectin.

There is, therefore, a necessity in finding out ways to eliminate or antagonize endogenously formed or exogenously administered TNF or, alternatively, to find ways to induce specifically only some of the many effects of TNF or restrict its effects to specific kinds of target cells. Our first attempt in this direction was the development of monoclonal antibodies which neutralize the TNF-α cytotoxic activity and were shown to protect mice against the lethal effect of TNF-α under conditions mimicking elicitation of septic shock, as described in Patent Application No. 73883 of the applicants.

Along the sequence of molecular events which take part in cell response to TNF, the most readily accessible to modulation is the initiation of this process, triggered by the binding of TNF to its cell surface receptors. One way to modulate this interaction is through induced changes in the expression of the TNF receptors. A different way of modulating the binding of TNF to its receptors is through the protective effect of the proteins TBP-I and TBP-II which bind TNF and thus decrease its availability to the TNF receptors.

The present invention provides antibodies specific for the two TNF-binding proteins TBP-I and TBP-II which have the following properties:

a) they block the effect of TNF on specific cells. This

blocking effect is obtained using the antibodies or F(ab) fragments thereof, taking advantage of the ability of said antibodies to block the binding of TNF to cells, apparently through interaction with structurally related cell surface TNF-binding proteins, i.e. TNF receptors; and

b) they mimic certain effects of TNF on specific cells. This mimicking effect of the antibodies is most likely due to activation of the receptors for TNF, upon their juxtaposition by the divalent antibodies, in a way which is similar to their activation by TNF itself. ——

The invention also comprises salts, functional derivatives and active fractions of the antibodies and of the F(ab) fragments thereof.

As used herein, the term "salts" refers both to salts of carboxyl groups and to acid addition salts of amino groups of the protein molecule.

"Functional derivatives" as used herein covers derivatives which may be prepared from the functional groups which occur as side chains on the residues or the N- or C- terminal groups, by means known in the art.

As "active fractions" of the antibodies and their F(ab) fragments, the present invention covers any fragment or precursors of the polypeptide chain of said protein molecules alone or together with associated molecules or residues linked

thereto, e.g. sugar or phosphate residues, or aggregates of the protein molecule or the sugar residues by themselves.

The antibodies of the present invention provide a new approach for the modulation of the TNF activity. They may be used both to inhibit and to mimic effects of TNF on specific subsets of cells, depending on the molecular form of the antibodies, specifically on their valence: monovalent forms of the antibodies (e.g. F(ab) fragments) being inhibitory and multivalent forms being able to mimic at least part of the effects of TNF.

The functional interaction of the antibodies of the present invention with cells provides also a new diagnostic tool, based on immunoassays such as radioimmunoassay, ELISA etc., for the detection of over- or under-production of TBPs by cells in the body in certain disorders. In an inverse approach, antibodies against the TBPs, when produced endogenously in the body, will be measured with the use of purified TBPs. Detecting such autoantibodies, when formed in certain autoimmune disorders, is of extreme importance, since their ability to mimic or inhibit the effects of TNF surely has far-reaching bearing on the pathological syndromes of said disorders.

The antibodies may be either polyclonal or monoclonal. They may be raised in rabbits, mice or other animals or tissue cultured cells derived thereof or can be products of cells of human origin. They may also be produced by recombinant DNA technology either in a form identical to that of the native antibody or as

chimeric molecules, constructed by recombination of antibody molecules of man and animal origins or in other forms chosen to make the antibodies most suitable for use in therapy.

For the preparation of the antibodies, either purified TBP-I or TBP-II or one or more synthetic peptides identical to the known sequences of each of the proteins, e.g. to the N-terminal protein sequences, may be used to immunize the animals. A further possibility is to fuse one of the possible nucleotide sequences deducted from the amino acid sequence of TBP-I or TBP-II to the gene coding for Protein A and to express the fused Protein A - TBP-I or Protein A-TBP-II gene in *E. coli*. The fused protein obtained is purified by affinity chromatography on IgG Sepharose column and then used to immunize the animals.

The monoclonal antibodies of the present invention are prepared using conventional hybridoma technique (Kohler et al. (1975) Nature 256:495; Kohler et al. (1976) Eur. J. Immunol. 6:511). After immunization, spleen cells alone or together with lymph node cells of the immunized animals are isolated and fused with a suitable myeloma cell line. After fusion, the resulting hybridoma cells are selectively maintained in HAT medium and then cloned. The hybridoma cells obtained through such a selection are then assayed to identify clones which secrete antibodies capable of binding TBP-I or TBP-II. After identification, the desired clones are grown in bulk, either in suspension culture or in ascitic fluid, by injecting the cells into the peritoneum of suitable host mice. The monoclonal antibodies produced by the hybridomas

are then isolated and purified.

The antibodies of the invention are found to inhibit the binding of TNF to cells. The two antibodies affect differently cells of different lines. Antisera to TBP-I block the binding of TNF to the cervical carcinoma HeLa cells and to the breast carcinoma MCF7 cells, but not to the histiocytic lymphoma U937 cells. Antisera against TBP-II have the inverse specificity. In the chronic myeloid leukemia K562 cells both antisera have inhibitory effects. These observations suggest that the TBPs are structurally related to the cell surface receptors for TNF and therefore that antisera against the TBPs can interact with the cell surface receptors in a way which can block the binding of TNF to cells. Furthermore, they indicate that there are two different TNF receptors and that cells of different lines express these two receptors in different proportions.

Consistently with these notions, F(ab) fragments of the anti-TBP-I antibodies are found to block the effect of TNF on cells of a line to which TNF binding is blocked by the antiserum comprising said antibodies. However, the intact antibody molecules are found to exert the inverse effect; they by themselves elicit in cells a cytotoxic effect identical to the effect induced by TNF. This ability of antibodies apparently directed against cell surface receptors to mimic the effect of the agonist, as opposed to the lack of ability of the F(ab) fragments of the antibody to do so, is mostly a consequence of the ability of the divalent antibody molecule to cause clustering

of the receptor molecules. This clustering somehow results in activation of the receptors in a way which is similar or identical to the activation of the receptors by TNF itself. Whatever the mechanisms involved, the fact that the antibodies can either mimic or inhibit the function of TNF, depending on the exact molecular form of the antibody applied, implies that these antibodies can practically serve as inhibitory or mimicking agents to TNF. Furthermore, since the antibodies to TBP-I and TBP-II bind to two different receptors for TNF, which are expressed on different cells exhibiting different responses to TNF and perhaps having also a different function even when expressed in the same cell, these inhibitory and mimicking effects of the antibodies to TBP-I and TBP-II can be applied for modulating the response to TNF, i.e. augmenting specifically beneficial effects and suppressing specifically deleterious effects of this cytokine.

The invention will now be illustrated by the following examples.

Example 1: Purification of the TNF binding proteins

Affinity purification on a column of immobilized TNF. Proteins of pooled urine of healthy postmenopausal women were concentrated 200 fold as described in Patent Applications No. 83878 and No. 90339. A sample of 250 ml of the concentrate was applied to a column constructed of 0.5 ml Affigel-10 to which 3.5 mg recombinant human TNF-α were coupled. The column was then washed with PBS (phosphate buffered saline) till all unbound proteins were removed and the bound proteins were eluted by applying a

solution containing 25 mM citric acid, 100 NaCl, 0.02% sodium azide at pH 2.5.

Reverse phase HPLC. The proteins eluted from the TNF affinity column were applied to an Aquapore RP300 column preequilibrated with 0.3% aqueous trifluoroacetic acid. The column was washed with the above solution at a flow rate of 0.5 ml/min, until all unbound protein was removed and then eluted with a gradient of acetonitrile concentrations in 0.3% aqueous trifluoroacetic acid. Two active components were obtained: TBP-I eluted at 27% acetonitrile and TBP-II at a somewhat higher concentration of acetonitrile (31%).

Example 2: Preparation of the antibodies against TBP-I and TBP-II

Immunization of rabbits with TBP-I and TBP-II obtained in Example 1 was, at doses of 5 µg and 20 µg respectively, in the following schedule: The proteins were first injected subcutaneously as emulsion in complete Freund adjuvant. Three weeks later they were injected again, intramuscularly, as emulsion in incomplete Freund adjuvant and then twice again subcutaneously as solution in PBS, at one week intervals. The rabbits were bled 10 days after the last immunization.

For the purification of immunoglobulins from the rabbit serum, saturated ammonium sulfate was added to 10ml serum to a final concentration of 50% saturation. After overnight incubation at 4°C, the immunoglobulins were precipitated by centrifugation. The pellet was washed twice with 50% ammonium sulfate, then

solubilized in 10mM sodium borate 0.02% sodium azide at pH 9. The solution was then dialyzed extensively against the borate-azide solution. It was then applied for chromatography on HPLC Mono-Q column, from which the proteins were eluted with a gradient of 0-500mM NaCl in the above borate-azide solution.

Example 3: Assays for the antibodies against TBP-I and TBP-II

Western blotting analysis. Tested proteins were applied to SDS PAGE on 10% acrylamide gels and then blotted electrophoretically to a nitrocellulose sheet. The nitrocellulose sheet was incubated with 10% milk (v/v) in PBS, then briefly rinsed in PBS and further incubated with the tested antibodies in a multi-lane device. After incubation with either ^{125}I-labelled Protein A or ^{125}I-goat antimouse F(ab) fragments of IgG, both at 5.10^5 CPM/ml, the unbound material was washed and the nitrocellulose sheet was exposed to autoradiography.

Figure 1 shows the Western blot analysis of the binding of antisera against TBP-I and TBP-II to the two proteins. TBP-I (A: lanes 1-6) and TBP-II (B: lanes 1-6) were applied to SDS PAGE at 2 µg/lane together with 2 µg BSA. Following electrophoresis the proteins were blotted electrophoretically to a nitrocellulose sheet which was then incubated with antiserum to TBP-I (lanes 1-3) or to TBP-II (lanes 4-6) at the following dilutions: lanes 1,4 - 1:100; lanes 2,5 - 1:500; lanes 3,6 - 1:2500. After incubation with the antibodies, the nitrocellulose sheet was incubated with ^{125}I-labelled protein A and then washed and exposed to autoradiography.

ELISA (Enzyme-linked immunosorbent assay). Ninety-six well ELISA plates were coated either with TBP-I or TBP-II or, as a control, with BSA (bovine serum albumin) at 1 µg/ml in PBS containing sodium azide by incubation. The wells were then rinsed with PBS-Tween, incubated with a solution of 0.5% BSA in PBS-Tween, and rinsed again and further incubated with the tested antibodies. After a further rinse, they were incubated with purified goat antibody to rabbit IgG, conjugated to horseradish peroxidase. The activity of horseradish peroxidase-conjugated antibody which bound to the plate was determined colorimetrically at 600 nM.

Figure 2 shows the results of ELISA for the binding of antisera against TBP-I and TBP-II to the two species of TBP. The binding of (□) antiserum against TBP-I to TBP-I, (■) antiserum against TBP-I to TBP-II, (o) antiserum against TBP-II to TBP-I, and (●) antiserum against TBP-II to TBP-II, is presented in terms of the absorbance of the color product of the enzymatic reaction applied in this test. The readings in a control test at which the antibodies were applied on wells coated with BSA were substracted.

Both figures 1 and 2 show that TBP-I and TBP-II are immunologically distinct (the slight binding of the antiserum against TBP-II to TBP-I, observed in Fig. 2, could be shown to be due to contamination of the antiserum with antibodies to TBP-I, at low amounts, due to the presence of some TBP-I in the preparation of TBP-II used for immunization).

Example 4: Effects of the antibodies on binding of TNF to cells

The antisera to TBP-I and TBP-II were diluted in PBS containing 0.5% BSA and 0.1% sodium azide and then either directly or, in competition experiments, after incubation with a sample of TBP, applied for 2 h on the tested cells of the HeLa, MCF7, K562 and U937 cell lines. The cells were then rinsed and tested for binding of TNF.

Figure 3 shows the inhibition of the ~~bin~~ding of radiolabelled TNF to U937, K562, HeLa and MCF7 cells with antisera to TBP-I (o) and TBP-II (□). The net binding observed in the absence of antisera (100%) was in U937 cells - 2500 CPM, in K562 cells - 1500 CPM, in HeLa cells - 2400 CPM and in MCF7 cells - 1100 CPM. The results demonstrate the fact that antisera against TBP-I and TBP-II interfere with the binding of TNF to cells; each affecting to a different extent cells of different lines. The antiserum against TBP-I inhibits effectively the binding of TNF to HeLa and MCF7 cells, but has no effect on the binding of TNF to U937 cells and only little effect on the binding of TNF to K652 cells. Inversely, the antiserum against TBP-II blocks effectively the binding of TNF to the K562 and U937 cells, but only at high concentrations inhibits the binding of TNF to the HeLa and MCF7 cells. Actually, its effect on the latter cell could be shown, by competition experiments, at which pure TBP-I and TBP-II were added to the serum, to be due just to the presence of contaminating antibodies to TBP-I in this preparation of antisera to TBP-II.

Example 5: F(ab) fragments of antibodies to TBP-I

Immunoglobulins purified from the antiserum to TBP-I were exposed to digestion with papain. The solution of the fragmented proteins was dialyzed against 10mM sodium acetate buffer, pH 5.5 and applied on HPLC Mono-S column. The bound proteins were eluted from the Mono-S column with a 0-300mM gradient of NaCl in the acetate buffer, which resulted in purification of the F(ab) fragments to homogeneity. To test the effect of these fragments on the cytocidal activity of TNF, they were applied on HeLa cells, the cells were then rinsed to remove all unbound antibodies and exposed for 10 h to TNF, at the indicated concentrations, together with cycloheximide (25 µg/ml). Viability of cells was then determined by the neutral red uptake method.

In Figure 4: (o) Viability of cells treated with the F(ab) preparation (at µg protein per ml). (●) Viability of cells which were not treated with the F(ab) preparation. The results show that the F(ab) fragments of the antibodies to TBP-I protect HeLa cells from the cytocidal effect of TNF - a reflection of the fact that they interfere with the binding of TNF to the TNF receptors expressed on the surface of these cells.

Table I shows that in the intact state antibodies to TBP-I have the inverse effect to that mediated by their F(ab) fragments; namely, they mimic the effect of TNF and are by themselves cytotoxic.

Table I

TNF-like cytocidal effect of antibodies to TBP-I

TNF (u/ml)	Cell Viability (%)
--	100
1	31
10	15
100	6
1000	3
anti TBP serum (dilution)	
1:6400	69
1:1600	37
1: 400	17
1: 100	9

TNF and the antiserum to TBP-I were applied on SV80 cells for 12 h together with cycloheximide. Viability of the cells was then determined by the neutral red uptake method.

Pharmaceutical compositions

The antibodies of the present invention can be formulated according to known methods to prepare pharmaceutical compositions, whereby either the purified antibody or the F(ab) fragment of the antibody are combined with a pharmaceutically acceptable carrier. Suitable vehicles and their formulation are described in Remington's Pharmaceutical Sciences by E.W. Martin. Such compositions will contain an effective amount of active substance hereof together with a suitable amount of vehicle for effective administration to the host. The amount of the active compound to be administered will depend on the route of administration, the disease to be treated and the condition of the patient.

The purified antibody or the F(ab) fragment thereof may be parenterally administered. Dosage and dose rate may parallel that currently in use in clinical investigations of other antibodies e.g. about 0.1-100 mg or even significantly elevated for greater effect.

Claims

1. An antibody to a human tumor necrosis factor binding protein designated TBP-I which specifically recognizes said protein.

2. An antibody as claimed in claim 1 which is further characterized in that it blocks the binding of TNF to HeLa and MCF7 cells.

3. An antibody as claimed in claim ~~1 or~~ 2 further characterized in that it does not block the binding of TNF to U937 cells.

4. An antibody according to claims 1 to 3 which is a monoclonal antibody.

5. An antibody according to claims 1 to 3 which is a polyclonal antibody.

6. F(ab) fragments of an antibody according to any of claims 1 to 5.

7. An antibody to a human tumor necrosis factor binding protein designated TBP-II which specifically recognizes said protein.

8. An antibody as claimed in claim 7 which is further characterized in that it blocks the binding of TNF to U937 and K562 cells.

9. An antibody as claimed in claim 7 or 8 further characterized in that it does not block the binding of TNF to HeLa and MCF7 cells.

10. An antibody according to claims 7 to 9 which is a monoclonal antibody.

11. An antibody according to claims 7 to 9 which is a polyclonal antibody.

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12. F(ab) fragments of an antibody according to any of claims 7 to 11.

13. The use of an antibody according to any of the preceding claims or of F(ab) fragments thereof or of salts, functional derivatives or active fractions of the antibody or of the fragment thereof, for blocking the binding of TNF to, and inhibiting its effect on cells.

14. The use according to claim 13 for the treatment of conditions wherein effects of TNF, either endogenously formed or exogenously administered, are to be antagonized.

15. The use of an antibody according to any of the preceding claims or of F(ab) fragments thereof or of salts, functional derivatives or active fractions of the antibody or of the fragment thereof, for mimicking beneficial effects of TNF on cells.

16. The use of claim 15 for mimicking the cytotoxic effect of TNF.

17. A quantitative assay for antibodies to TBP-I or TBP-II endogenously produced in man in autoimmune diseases, by measuring their binding to the proteins TBP-I or TBP-II, respectively.

18. An immunoassay for the TNF binding proteins TBP-I and TBP-II in body fluids characterized by measuring their interaction with an antibody according to claim 1 or claim 7, respectively.

For the applicants

Paulina Ben-Ami
Patent Attorney

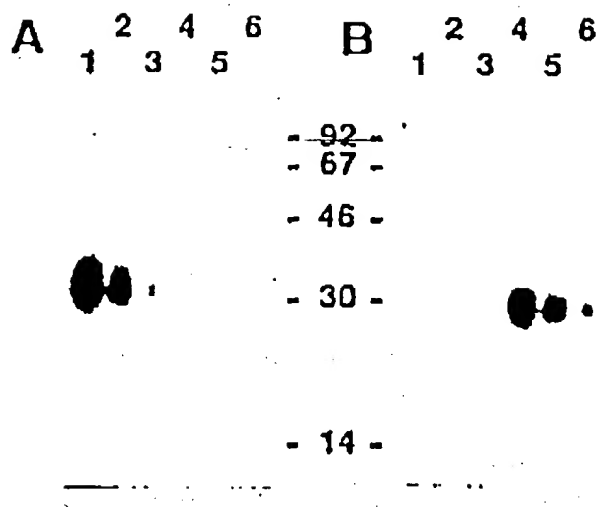

FIGURE 1

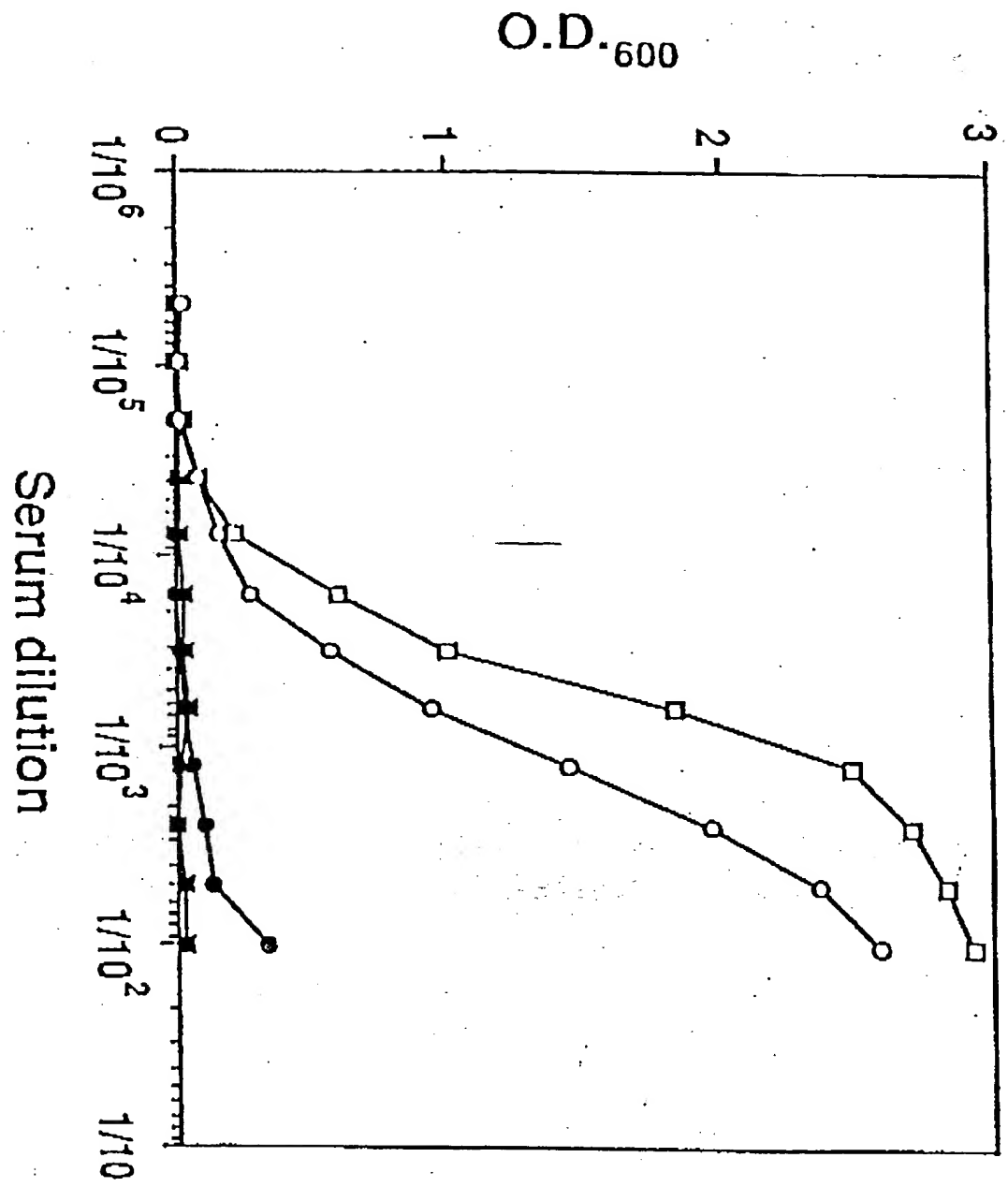

FIGURE 2

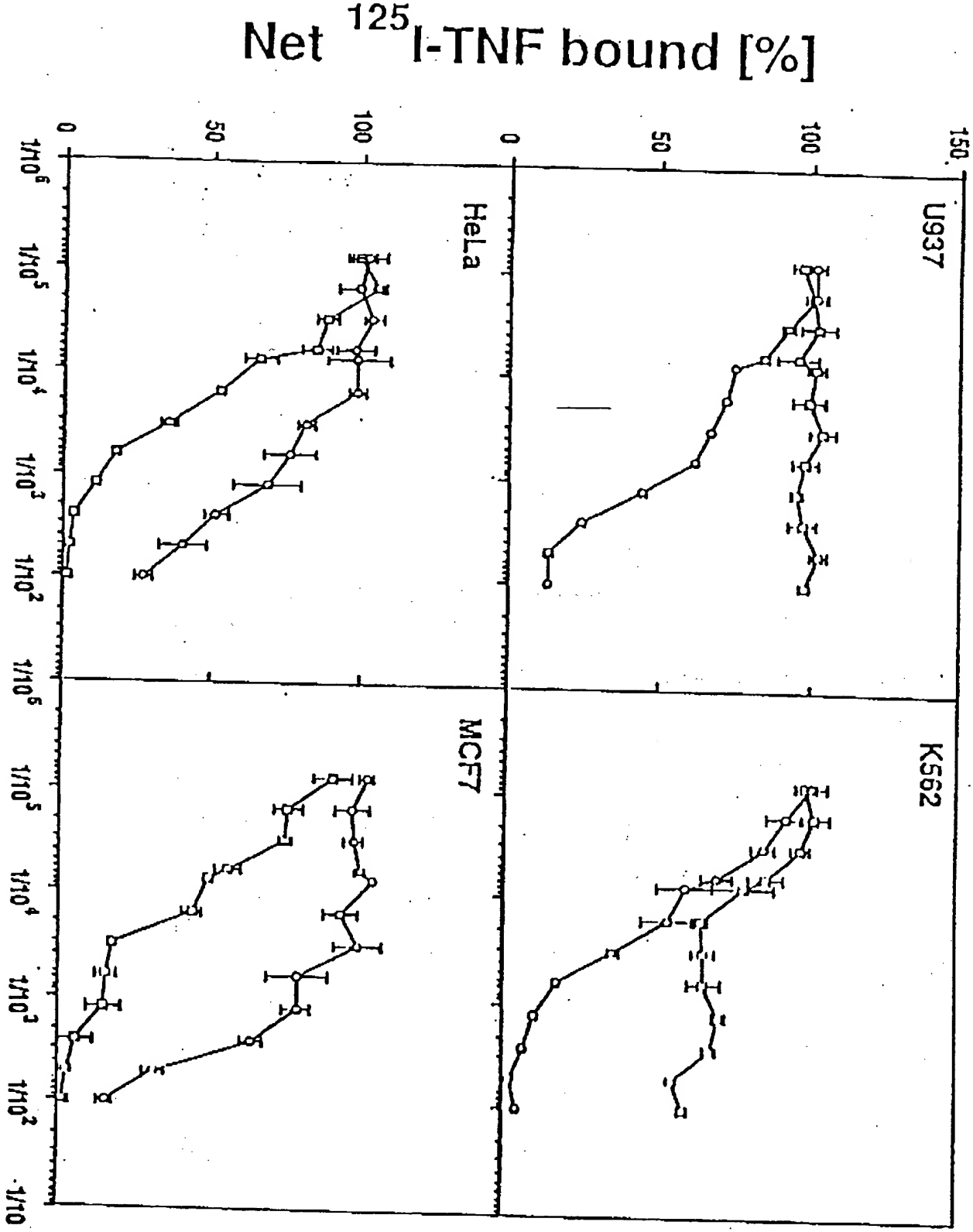

FIGURE 3

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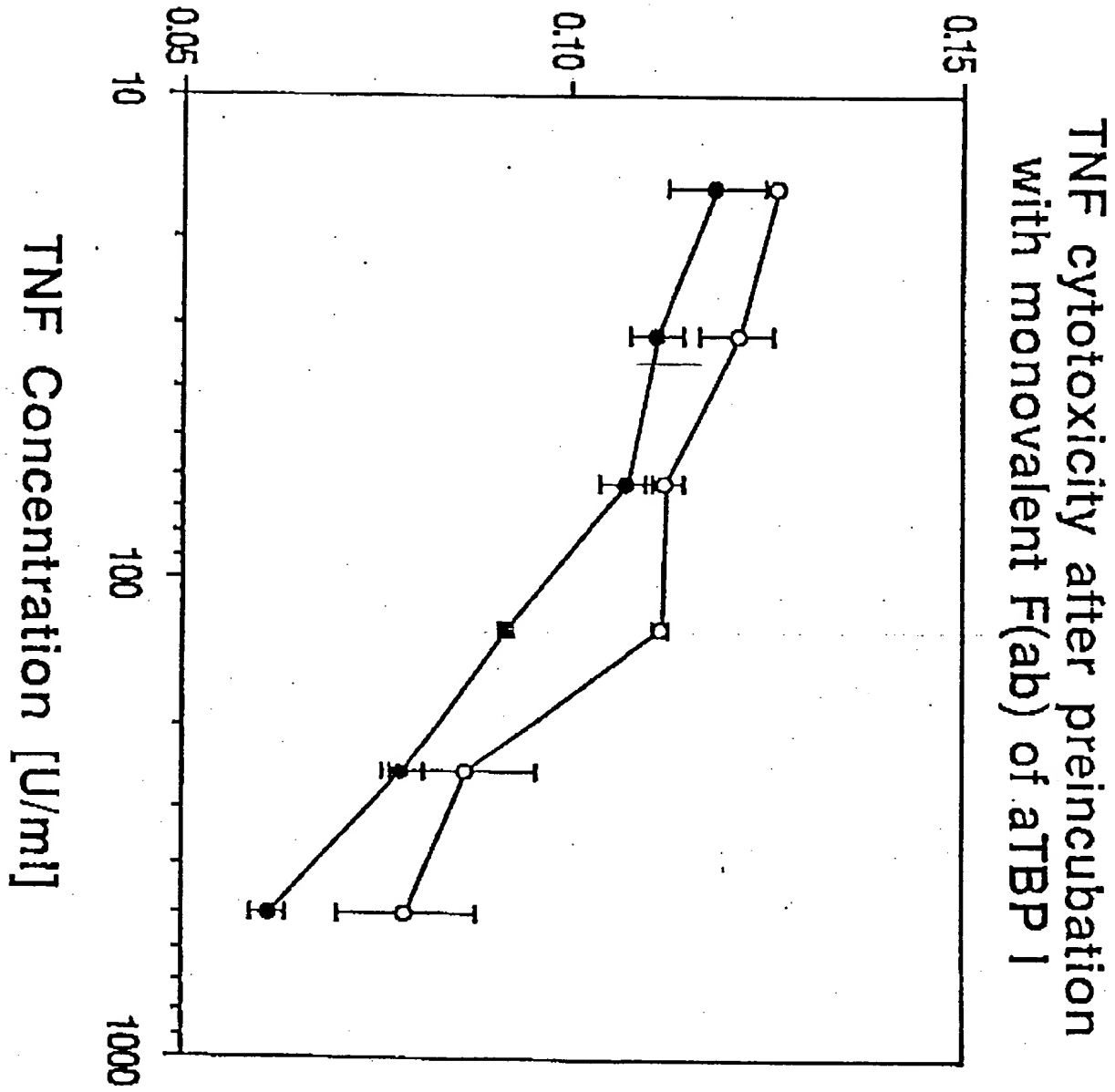

FIGURE 4

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